propanediol dehydrase reactions (Zagalak *et al.*, 1966) inversion of substrate configuration occurs, whereas in the methylmalonyl-CoA mutase (Sprecher *et al.*, 1966b) and ribonucleotide reductase of *L. leichmannii* reaction the configuration of substrate is retained.

## References


Abeles, R. H., and Zagalak, B. (1966), *J. Biol. Chem. 241*, 1245.

Blakley, R. L., Ghambeer, R. K., Batterham, T. J., and Brownson, C. (1966), *Biochem. Biophys. Res. Commun. 24*, 418.

Conroy, H. (1960), *Advan. Org. Chem. 2*, 265.

Gatlin, L., and Davis, J. C. (1962), *J. Am. Chem. Soc. 84*, 4464.

Gunsalus, J. C., and Razzell, W. E. (1957), *Methods Enzymol. 3*, 941.

Ingold, C. K. (1953), Structure and Mechanism in Organic Chemistry, Ithaca, N. Y., Cornell University, p 380.

Jardetzky, C. D. (1960), *J. Am. Chem. Soc. 82*, 299.

Karplus, M. (1963), *J. Am. Chem. Soc. 85*, 2870.

Kokko, J. P., Goldstein, J. H., and Mandell, L. (1961), *J. Am. Chem. Soc. 83*, 2909.

Lemieux, R. U. (1961), *Can. J. Chem. 39*, 116.

Miles, H. T. (1963), *J. Am. Chem. Soc. 85*, 1007.

Miles, H. T., Bradley, R. G., and Becker, E. D. (1963), *Science 142*, 1569.

Orr, M. D., and Vitols, E. (1966), *Biochem. Biophys. Res. Commun. 25*, 109.

Sprecher, M., Clark, M. J., and Sprinson, D. B. (1966b), *J. Biol. Chem. 241*, 872.

Sprecher, M., Switzer, R. L., and Sprinson, D. B. (1966a), *J. Biol. Chem. 241*, 864.

Sundaralingam, M. (1965), *J. Am. Chem. Soc. 87*, 599.

Weissbach, H., and Dickerman, H. (1965), *Physiol. Rev. 45*, 80.

Zagalak, B., Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1966), *J. Biol. Chem. 241*, 3028.


# The Isolation and Identification of L-Lanthionine and L-Cystathionine from Insect Haemolymph*

D. Rajagopal Rao,† A. H. Ennor, and B. Thorpe

ABSTRACT: Lanthionine and cystathionine have been isolated in crystalline form from the deproteinized haemolymph of *Bombyx mori* (silkworm) and *Antheraea pernyi* (Japanese oak moth) by ion-exchange chromatography. Both compounds have been characterized as the L-enanthiomorphs by enzymatic and physical methods. A preliminary survey of the distribution of lanthionine and cystathionine throughout a number of phyla has been carried out and the results of this, together with some preliminary experiments on the *in vivo* incorporation of isotopically labeled materials, are reported.

The presence of free L-lanthionine in insect issues is invariably associated with a complete absence or, at best, barely detectable traces of cysteine, cystine, and methionine.

During the course of investigations into the metabolism of certain amino acids in insects, the presence of two unusual amino compounds was detected by paper chromatography in the haemolymph of *Bombyx mori* (silkworm) and *Antheraea pernyi* (Japanese oak moth). The compounds have been identified as L-lanthionine and L-cystathionine and the present report deals with their detection, isolation, and characterization. In addition, the results of some experiments on the amino acid composition of haemolymph from *A. pernyi* together with preliminary experiments on the distribution of lanthionine and cystathionine are reported together with some results of isotopic incorporation studies.

## Experimental Section

*Materials.* All reagents were of analytical grade and solvents used for chromatography were purified by fractional distillation. *meso*-Lanthionine and D- and L-cystathionine were gifts from Dr. D. McHale of Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey, U.K., and Dr. N. Horowitz of

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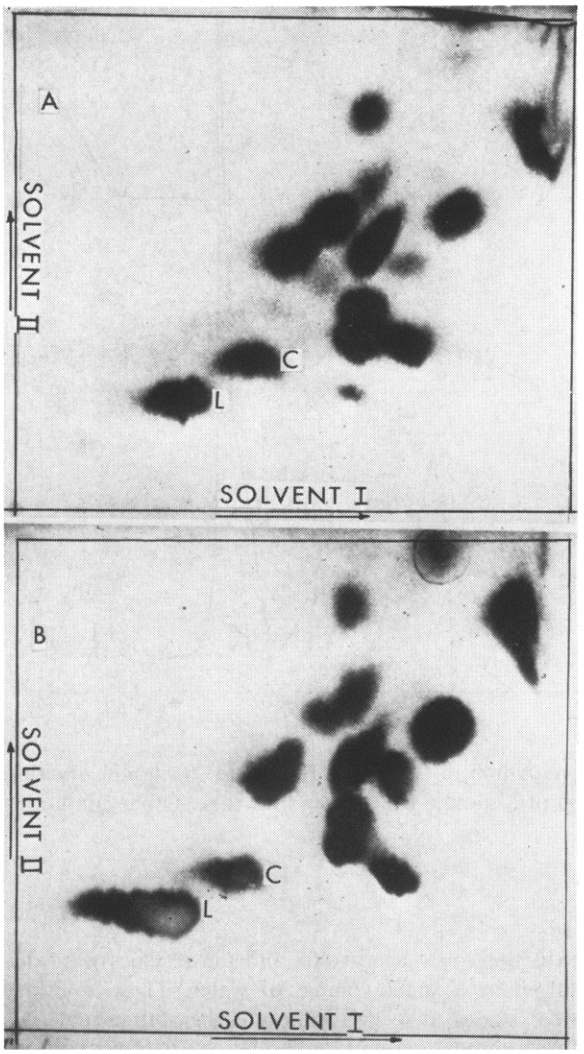


FIGURE 1: Two-dimensional chromatograms of a protein-free extract of haemolymph of (A) *B. mori* and (B) *A. pernyi*. Experimental conditions as described in the text. Spots C and L represent the unknown compounds.

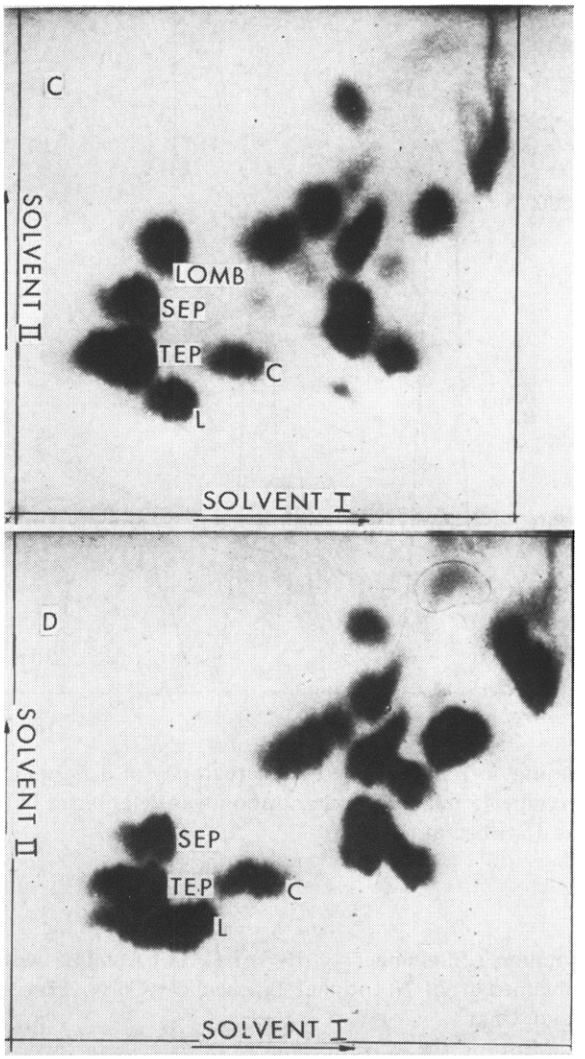


FIGURE 2: Two-dimensional chromatograms of deproteinized haemolymph of (C) *B. mori* with added markers of lombricine (LOMB), serine ethanolamine phosphodiester (SEP), and threonine ethanolamine phosphodiester (TEP); (D) deproteinized haemolymph of *A. pernyi* with added markers of SEP and TEP.

California Institute of Technology, respectively. Lanthionine was also synthesized according to the method of Schöberl and Wagner (1947). The product was a mixture of the *meso*- and L-enantiomorphs from which the L isomer was separated by a series of fractional crystallizations from hot water. The synthetic L-lanthionine was estimated to contain 5% of the *meso*-enantiomorph by analysis on the Beckman amino acid analyzer, treatment with L-amino acid oxidase, and by infrared spectroscopy.

L-[$^{35}$S]cystine, L-[$^{35}$S]methionine, and L-[3-$^{14}$C]serine were purchased from the Radiochemical Centre, Amersham, U.K. They were checked for presence of radioactive impurities and purified by preparative paper chromatography.

The following ion-exchange resins were used: Dowex 50W-X4, $H^+$ form (100–200 mesh); Dowex 50W-X4 $NH_4^+$ form (100–200 mesh); and Amberlite CG-120 (type 1), $H^+$ form (100–200 mesh). Before use all resins were repeatedly recycled by passage through the $Na^+$ and $H^+$ or $NH_4^+$ and $H^+$ forms and exhaustively washed with water.

D-Amino acid oxidase was prepared from hog kidneys and carried up to stage III of the preparation described by Massey *et al.* (1961). L-Amino acid oxidase was prepared by dissolving 0.5 g of dried *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) in 10 ml of 0.01 M Tris-HCl buffer, pH 7.2. This preparation was used after dialysis for 24 hr at 2° against 200 volumes of the same buffer

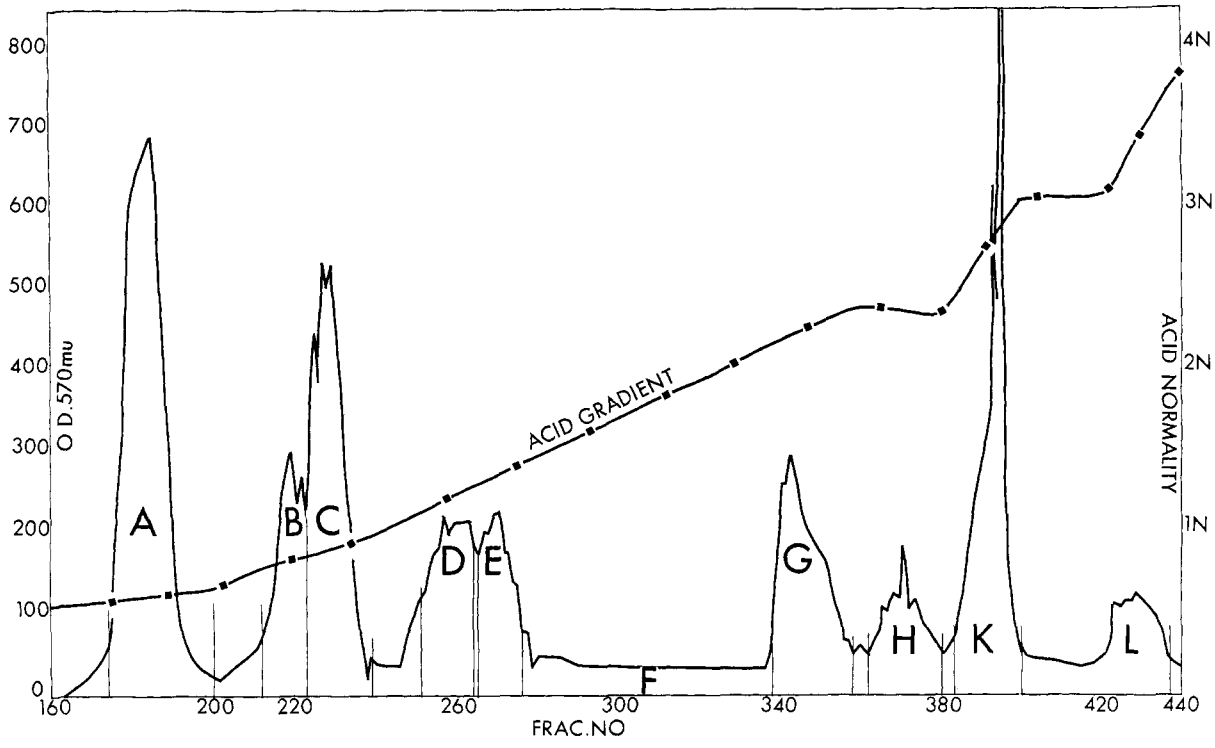


FIGURE 3: Ion-exchange chromatography of deproteinized haemolymph of *A. pernyi*. Flow rate 20 ml/min, fraction volume 45 ml. Acid concentration was determined by titration of a sample from every fifth tube. Other conditions as described in text.

solution containing $5 \times 10^{-7}$ M FAD.[1] Catalase was obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Most of the experimental work has been carried out with haemolymph from 10-day-old pupae of laboratory reared *B. mori* and from *A. pernyi* in the diapause phase. The latter material together with many specimens of other insects was a gift from Dr. T. D. R. Grace, Division of Entomology, C.S.I.R.O., Canberra.

*Methods.* PREPARATION OF PROTEIN-FREE EXTRACTS. The haemolymph of *B. mori* and *A. pernyi* was deproteinized by the addition of an equal volume of 0.5 N perchloric acid. Other specimens were disintegrated in a "Nalco" homogenizer in the presence of an equal volume of 0.5 N perchloric acid or ground to a fine powder in liquid nitrogen and suspended in an approximately equal volume of 0.5 N perchloric acid. The precipitated protein was removed by centrifugation and the extracts, after chilling to 2°, were adjusted to pH 7.0 by addition of 5 N KOH. The precipitated $KClO_4$ was removed and the extracts were adsorbed on columns of Dowex 50-$H^+$. The columns were then washed with water until the effluent was neutral after which the adsorbed material was eluted with 2.5 N $NH_4OH$. The elutes were evaporated to dryness *in vacuo* at 40° and the residues were dissolved in a small volume of water. These solutions were stored at −10° and were the source materials for all chromatographic and isolation experiments.

PAPER CHROMATOGRAPHY AND ELECTROPHORESIS. Ascending two-dimensional paper chromatograms (Whatman No. 3MM) were developed in ethanol–formic acid (98–100%)–water (7:1:2, v/v) as solvent I and in phenol–water (4:1, v/v) as solvent II. Ascending unidimensional paper chromatograms were developed in 1-butanol–acetic acid–water (12:3:5, v/v) (solvent III), *t*-butyl alcohol–ethyl methyl ketone–ammonium hydroxide–water (10:10:3:5, v/v) (solvent IV), phenol (solvent II)–ethanol–ammonium hydroxide (15:4:1, v/v) (solvent V), and phenol (solvent II)–ammonium hydroxide (200:1, v/v) (solvent VI).

Samples were subjected to electrophoresis for 10 min at 7500 v on Whatman 3MM paper (38 × 54 cm) in an apparatus similar to that described by Rothman and Higa (1962). Chromatograms and electropherograms were dried, sprayed with ninhydrin (0.2% w/v in acetone), and heated for 10 min at 80° to detect amino acids.

ION-EXCHANGE CHROMATOGRAPHY. Fractionation of amino acids on a column 4.5 × 80 cm of Amberlite CG-120-$H^+$ with an increasing concentration gradient of $HClO_4$ was carried out essentially as described by Hurlbert *et al.* (1954). The mixing vessel contained 1 l. of 0.06 N $HClO_4$ and the reservoir initially

[1] Abbreviation used: FAD, flavin-adenine dinucleotide.

contained 2 l. of 0.12 N $HClO_4$. When this solution was exhausted, it was replaced successively by 2 l. each of 0.24, 0.48, 0.72, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 N $HClO_4$. The effluent was collected in 45-ml fractions at a flow rate of 11 ml/min. Ninhydrin-reacting material contained in each fraction was detected as described by Rosenberg *et al.* (1962). The amino acids in each fraction were subsequently identified by paper chromatography and by a Beckman automatic amino acid analyzer (Model 120B) using a 50-cm column on the accelerated system; a 30–50° schedule was followed with the temperature change at 2.5 hr and the buffer change at 2 hr and 40 min. L-Lanthionine is eluted just before proline under these conditions.

Protein-free extracts of most of the specimens have also been examined on the Technicon amino acid analyzer (column dimensions 0.6 × 150 cm, grade "A" chromobeads, spherical resin, temperature 60°, flow rate 30 ml/hr, and duration of analysis 21 hr) using the standard buffer system as recommended by the manufacturers. L-Lanthionine and *meso*-lanthionine are eluted after alanine under these conditions. Total amino acids were estimated against an alanine standard, by the ninhydrin method of Rosen (1957), and proline by the method of Piez *et al.* (1956).

In those experiments where radioisotopes were used 10–25 μl of an aqueous solution of the appropriate compound (L-[$^{35}$S]cystine, L-[$^{35}$S]methionine, or L-[3-$^{14}$C]serine) was injected into 6-day-old pupae of *B. mori* and the site of injection was sealed with paraffin wax. After various time intervals, haemolymph samples were taken or the entire pupae were ground to a powder under liquid nitrogen, deproteinized with perchloric acid, and the total free amino acid pool was isolated as described earlier. Samples of these extracts were examined on the Beckman amino acid analyzer using a flow cell of the scintillation counter in the effluent stream before the manifold of amino acid analyzer (Schram, 1963; Spackman *et al.*, 1958).

## Results

Examination of the protein-free extracts of haemolymph from both *B. mori* and *A. pernyi* by two-dimensional paper chromatography showed the presence of two unknown ninhydrin-reacting compounds of low $R_F$ similar to those of serine and threonine ethanolamine phosphates and lombricine. When these compounds were added to the material to be chromatographed there was, however, a clear separation (Figures 1 and 2).

Small amounts of each compound were purified by paper chromatography and subjected to a number of spot tests. Neither compound contained SH or SS linkages as indicated by negative nitroprusside tests both before and after treatment with cyanide. Both gave a positive result with iodoplatinic reagent (Smith, 1960) and on fusion with metallic sodium (Feigl, 1960) both were shown to contain sulfur. It was, therefore, assumed that each compound contained thioether linkages. To permit further characterization a large-scale isolation of the compounds by ion-exchange chromatography was carried out. A deproteinized and desalted extract of haemolymph (60 ml) from *A. pernyi* was prepared as described in Methods. After evaporation of the ammoniacal eluate the residue was dissolved in 80 ml of water and the solution was subjected to ion-exchange chromatography on Amberlite CG-120-$H^+$. Nine main ninhydrin-positive peaks were obtained (Figure 3) and the contents of those tubes constituting these peaks were combined. These nine fractions were desalted and examined by paper chromatography; the amino acid content of each is given in Table I. Those fractions containing peaks G and H were found to contain the unidentified sulfur-containing amino compounds together with small amounts of ninhydrin-reacting contaminants. The solutions containing peaks G and H (amounting to about 900 and 630 ml, respectively) were passed through columns of Dowex 50-$H^+$. The adsorbed material which constituted these peaks was then eluted with 2 N $NH_4OH$, and the eluates were evaporated to dryness under reduced pressure. Both residues were dissolved in about 20 ml of water. One-half of each solution was then transferred to columns (2.8 × 68

TABLE I: Composition of Amino Acid Peaks from Amberlite Fractionation of Haemolymph Extracts from *A. pernyi*.[a]

| Peak | Amino Acids Present | μmoles Recovd |
|---|---|---|
| A | Glutamic + serine + threonine | 1095 |
| B | Glycine + alanine (trace) | 535 |
| C | Alanine | 665 |
| D | Valine + leucine (trace) | 301 |
| D + E | Valine + leucine + proline | 272 |
| E | Proline + leucine (trace) | 736 |
| G | Lanthionine + phenylalanine (trace) | 460 |
| H | Cystathionine + tyrosine (trace) | 251 |
| K | Lysine + histidine | 815 |
| L | Arginine | 375 |
| Total | | 5485 |
| Starting material (% recov, 87) | | 6368 |

[a] The amino acids were identified by paper chromatography and electrophoresis and the amounts present were determined as described in the text.

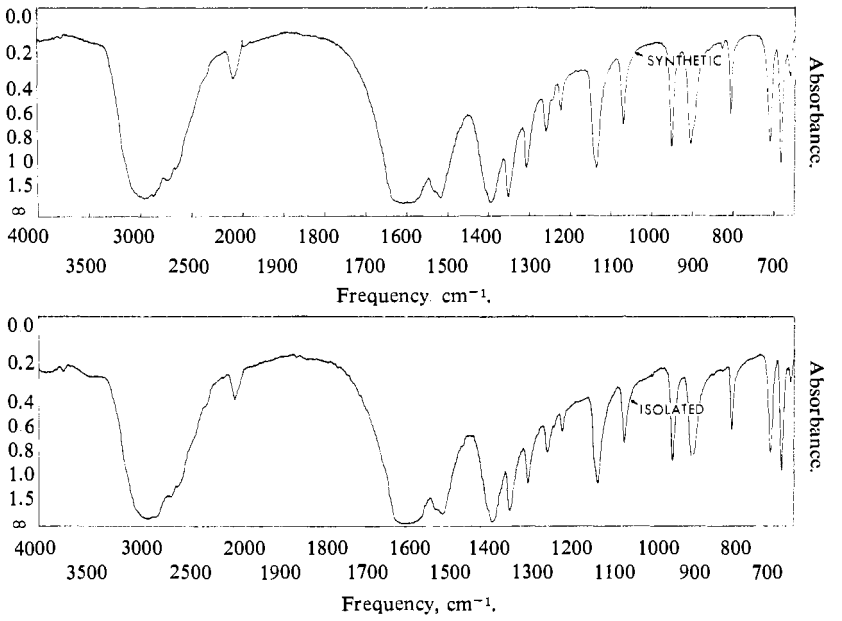


FIGURE 4: Infrared spectra of synthetic and isolated L-lanthionine recorded on Perkin-Elmer Model 21 spectrophotometer.

TABLE II: Specific Rotations[a] of Isolated Lanthionine and Synthetic[b] L-Lanthionine.

| Wavelength (mμ) | 2% (w/v) in 1.0 N HCl | | 2% Aq (w/v) Containing 1 Equiv of NaOH | | 1% (w/v) in 1.0 N NaOH | |
|---|---|---|---|---|---|---|
| | Isolated | Synthetic | Isolated | Synthetic | Isolated | Synthetic |
| 313 | +124.25 | +112.53 | −57.7 | −57.97 | +75.0 | +75.75 |
| 364 | +47.25 | +40.34 | −57.0 | −57.97 | +36.0 | +32.0 |
| 436 | +18.50 | +14.70 | −44.75 | −44.85 | +16.00 | +13.25 |
| 546 | +6.50 | +3.41 | −29.0 | −28.59 | +7.50 | +6.30 |
| 578 | +5.00 | +2.36 | −26.0 | −25.44 | +8.00 | +4.20 |
| 589 | +5.75 | +2.36 | −24.70 | −24.92 | +5.00 | +4.20 |

[a] Optical rotations were measured in a 2-cm tube at 22° using a Perkin-Elmer polarimeter, type 141. [b] Values have been corrected for a 5% contamination of the synthetic sample by *meso*-lanthionine.

cm) of Dowex 50-$NH_4^+$, eluted with water, and the eluate was collected in 5-ml fractions at a flow rate of 8 ml/min. Tubes containing ninhydrin-reacting compounds (tubes 39–59, peak G; and 64–106, peak H) were examined chromatographically. Tubes 39–54 (peak G) showed a single ninhydrin-reacting component which corresponded to one of the unknown sulfur-containing compounds. These tubes were combined, evaporated *in vacuo* to approximately 2 ml, and then transferred to the cold room (2°) when crystallization commenced. The crystals (20 mg of white platelets) were recovered by filtration and dried *in vacuo* over $P_2O_5$. Tubes 75–97 (peak H) contained the other unknown sulfur compound, which was isolated as described for peak G, yielding 8 mg of crystals. In another experiment with 155 ml of *A. pernyi* haemolymph as starting material, 221 mg of lanthionine and 35 mg of cystathionine were isolated.

*Identification of Peak G as* L-*Lanthionine. Anal.* Calcd for $C_6H_{12}N_2O_4S$: C, 34.61; H, 5.80; N, 13.46; S, 15.40. Found (isolated): C, 34.63; H, 5.97; N, 13.64; S, 15.20; and (synthetic): C, 34.77; H, 5.92; N, 13.38; S, 15.56.

*Raney Nickel Degradation.* A sample of fraction G was subjected to Raney Nickel degradation as described by Berridge *et al.* (1952). The reaction mixture was desalted on Dowex 50-$H^+$ and examined by high-voltage paper electrophoresis and unidimensional paper chromatography in solvents III–VI. Only one ninhydrin-reacting spot was detected and this corresponded in mobility to alanine. It was, therefore, assumed that peak G was lanthionine. An authen-

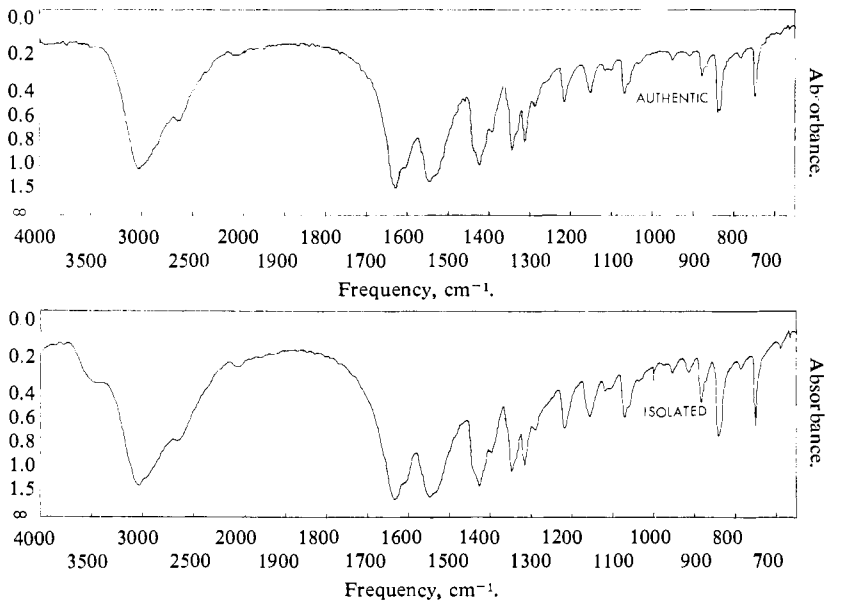


FIGURE 5: Infrared spectra of authentic and isolated L-cystathionine recorded on Perkin-Elmer Model 21 spectrophotometer.

tic sample of lanthionine when subjected to similar treatment gave identical results.

Measurement of optical rotation under varying conditions (Table II) showed the isolated and synthetic samples to possess similar properties. Infrared analyses (KBr pellet) were carried out on isolated and synthetic samples of lanthionine (Figure 4). Synthetic and isolated samples gave identical absorption maxima (360 and 450 mμ) with acid ninhydrin (Work, 1957).

Incubation of synthetic L-lanthionine and the isolated material with excess D-amino acid oxidase and catalase in pyrophosphate buffer, 0.03 M at pH 8.3, containing FAD ($10^{-5}$ M) at 37° resulted in no $O_2$ uptake over a period of 6 hr. Reaction mixtures were deproteinized, desalted, and analyzed on the amino acid analyzer. Recoveries of 94 and 92% of the starting material were obtained for isolated and synthetic samples, respectively.

However, treatment of materials with L-amino acid oxidase and catalase at 37° at pH 7.2 in 0.03 M Tris-HCl buffer, resulted in $O_2$ uptake. After 6-hr incubation no residual lanthionine could be detected in the deproteinized reaction mixtures.

*Identification of Peak H as* L-*Cystathionine. Raney Nickel Degradation.* The procedure employed was precisely similar to that described above for lanthionine. Two reaction products were detected chromatographically and these were identified as alanine and α-amino-*n*-butyric acid. An authentic sample of cystathionine gave similar reaction products and it was concluded that fraction H was either cystathionine or β-methyllanthionine. The infrared spectra (KBr pellet) of the isolated material and authentic cystathionine were identical (Figure 5). Optical rotation measured at various wavelengths gave identical results for the isolated material and authentic L-cystathionine (Table III). Authentic and isolated samples gave identical absorption maxima (340 mμ) with acid ninhydrin (Work, 1957). Enzymic experiments with L- and D-amino acid oxidases showed the isolated material was completely oxidized by L-amino acid oxidase and it was unaffected by D-amino acid oxidase.

The results of experiments with possible $^{35}S$- and $^{14}C$-labeled precursors of lanthionine and cystathionine are given in Table IV. $^{35}S$ from methionine is incorporated more rapidly into cystathionine than into lanthionine. A similar result is obtained in the case of L-

TABLE III: Specific Optical Rotation[a] of Isolated Cystathionine and Synthetic L-Cystathionine in 1.0 N HCl[b] at 22° at Varying Wavelengths.

| Wavelength (mμ) | Isolated | Synthetic |
|---|---|---|
| 313 | +178.75 | +187.20 |
| 364 | +96.25 | +99.75 |
| 436 | +53.50 | +55.25 |
| 546 | +29.00 | +29.00 |
| 578 | +25.50 | +25.00 |
| 589 | +25.50 | +25.00 |

[a] Optical rotations were measured in a 2-cm tube at 22° using a Perkin-Elmer polarimeter, type 141. [b] The concentration of cystathionine (synthetic and isolated) was 20 mg/ml.

TABLE IV: Incorporation of $^{35}S$ and $^{14}C$ into Lanthionine and Cystathionine[a] in *B. mori* pupae.

| No. | Isotope Injected | Duration of Expt (hr) | Amino Acid | Amt (μmole) | Sp Act. (cpm × $10^3$/μmole) |
|---|---|---|---|---|---|
| 1 | L-[$^{35}S$]Methionine | 6 | Lanthionine | 0.17 | 7 |
| | | | Cystathionine | 0.07 | 64 |
| | | | Methionine | 0.25 | 1300 |
| 2 | L-[$^{35}S$]Methionine | 24 | Lanthionine | 0.13 | 21 |
| | | | Cystathionine | 0.03 | 410 |
| | | | Methionine | 0.02 | 425 |
| 3 | L-[$^{35}S$]Cystine | 6 | Lanthionine | 0.26 | 48 |
| | | | Cystathionine | 0.18 | 52 |
| | | | Cystine | None | – |
| 4 | L-[$^{35}S$]Cystine | 24 | Lanthionine | 0.34 | 109 |
| | | | Cystathionine | 0.11 | 40 |
| | | | Cystine | None | – |
| 5 | L-[$^{14}C$]Serine | 6 | Serine | 0.45 | 31 |
| | | | Lanthionine | 0.24 | 1 |
| | | | Cystathionine | 0.22 | 9 |

[a] In expt 1 and 2, samples of the total amino acid pools from the entire pupae were used for analyses. In expt 3–5, samples of the amino acid fractions from the haemolymphs were used for analyses. Pupae (6-day-old) were used in expt 1–4; 11-day-old pupae in expt 5. The amount of isotope injected was approximately 1 × $10^6$ cpm except in the case of expt 5 where 2 × $10^5$ cpm were injected.

[3-$^{14}C$]serine. The injection of L-[$^{35}S$]cystine is followed by rapid incorporation of isotope into both compounds and none of the injected cystine could be detected after a 6-hr period.

Some experiments have been carried out on the distribution of both lanthionine and cystathionine and protein-free extracts of specimens from members of various phyla have been examined. Both amino acids were found only in several members of the Insecta and those giving positive results are listed in Table V. Except where noted, neither lanthionine nor cystathionine were present in the following: Mammalia, mouse (small intestine); Reptilia, *Amphiboluris diemensis* and *Egemia cunninghami* (liver, small intestine, and skeletal muscle from adult specimens–cystathionine present in all samples); Amphibia, *Bufo marinus* (liver–entire intestinal tract and skeletal muscle–cystathionine present in all samples); Mollusca, extracts of entire organisms, *Subninella undulata*, *Modiolus cottoni*, *Ischnonchiton versicolor*, *Ostrea angasi*, and *Scutus antipodes* (cystathionine present in latter three); Annelida, *Sabellastarte indica* and *Megascolides cameroni;* Urochordata, *Pyura stolonifera;* Coelenterata, *Actinia tenebrosa;* Echinodermata, *Ophionereus scehayei*, *Patiriella calca*, and *Heliocidaris erythrogramma* (cystathionine present in latter two); Arthropoda, *Postunus pelagicus* (material showing chromatographic behavior of cystathionine present).

## Discussion

Lanthionine was first isolated by Horn *et al.* (1941) from the acid hydrolysates of alkali-treated wool. It was later shown that other proteins, if treated first with alkali and then subjected to acid hydrolysis, also gave rise to lanthionine (Horn and Jones, 1941; du Vigneaud *et al.*, 1941; Horn *et al.*, 1942a,b; Dowling and McClaren, 1965). In these cases it was believed that the lanthionine was an artifact arising from the decomposition of cysteine residues.

The amino acid composition of insect haemolymph from various genera has been investigated by several workers and has recently been reviewed (Wyatt, 1961; Chen, 1962). L-Cystathionine was previously isolated from the haemolymph of *B. mori* (Kondo, 1959) but was reported to be absent in *A. pernyi* (Kondo, 1962).

The present work has revealed that L-lanthionine and L-cystathionine are present as free amino acids in the haemolymph of both *B. mori* and *A. pernyi* and that in the case of the former both compounds are present throughout the various developmental stages. Since neither amino acid could be detected in extracts of mulberry leaf and since these leaves were the sole diet of *B. mori*, it is presumed that they are the products of biosynthetic reactions.

The present work has also been concerned with a study of the distribution of both lanthionine and cystathionine and has revealed that they are widely though not universally distributed throughout members of the Insecta. In no case was it possible to demonstrate the presence of lanthionine without the concomitant presence of cystathionine and in those cases where both were present there was rarely any measurable

TABLE V: Distribution of Lanthionine and Cystathionine in Various Insects.[a]

| Organism | | |
|---|---|---|
| Class | Species | Stage Examined |
| | Insecta | |
| Coleoptera | *Tenebrio molitor* | Larvae |
| | *Sericesthis pruinosa* | Larvae |
| Lepidoptera | *Tineola bisellnella* | Pupae |
| | *Bombyx mori* | Moth, eggs, pupae, larvae |
| | *Antheraea pernyi* | Pupae |
| | *Antheraea Helena* | Larvae |
| | *Antheraea eucalypti* | Larvae |
| | *Antheraea mollitor* | Pupae |
| Diptera | *Lucilla cuprina* | Larvae |
| Lampyridae | *Photinus pyralis*[b] | Adult |

[a] Other insects which have been examined and which do not contain lanthionine and cystathionine are: Coleoptera, *Anthrenus, Phoracantha semipunctata, Aulacocylas collaris* (Blackburn); Orthoptera, *Periplaneta americana* (Cystathionine only present); Isoptera, *Nasutitermes exitiosis;* Lepidoptera, *Galleria melonella.* [b] Lanthionine was isolated from extracts of whole specimens and was characterized as the L-enanthiomorph as described in the text for *A. pernyi.*

amount of cysteine, cystine, or methionine. These observations together with the structural similarity of lanthionine and cystathionine are suggestive of some novel pathways of sulfur metabolism and this possibility is being investigated.

The presence of quite large amounts of lanthionine and cystathionine as free amino acids in the haemolymph has suggested the possibility that one or both amino acids may also be present in the proteins. Such an indication indeed also stems from the work of Stein (1955) and Stein and Kermack (1959) who reported lanthionine to be present in the protein of the wing muscle of locusts. In our experiments we have not been able to demonstrate either lanthionine or cystathionine in the acid hydrolysates of protein derived from the haemolymph of *B. mori, A. pernyi,* and *Antheraea eucalypti.* Some experiments have been carried out with perchloric acid and trichloroacetic acid extracted material from *B. mori* (pupae) and *Photinus pyralis* (adult). This material, from which all free amino acids were extracted, showed, after acid hydrolysis, no trace of lanthionine. In this connection the recently reported work of Sloane and Untch (1966) is of interest for these workers have demonstrated not only the presence of L-lanthionine as a free amino acid in chick embryos but also its presence in acid hydrolysates of chick embryo protein. An enzyme system in the yolk of the hen's egg which catalyzes the formation of lanthionine from cysteine has been described by Chapeville and Fromageot (1961).

Sloane and Untch (1966) have pointed out that the infrared spectrum of L-lanthionine was similar to that earlier reported by us (Rajagopal Rao *et al.*, 1966) but that their observed direction of rotation while similar to that reported by Brown and du Vigneaud (1941) and Schöberl and Wagner (1947) was opposite to that which we reported. This apparent divergency in findings is explained by the fact that the observed direction of rotation varies with the ionization state of lanthionine (*cf.* Table II). A similar influence of the ionization state has been observed by other workers in case of other amino acids (Billardon, 1960; Katzin and Gulyas, 1964). The optical rotatory dispersion of some sulfur containing amino acids is also particularly influenced to varying degrees by temperature (Bláha *et al.*, 1965).

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References

Berridge, N. J., Newton, G. G. F., and Abraham, E. J. (1952), *Biochem. J. 52,* 529.

Billardon, M. (1960), *Compt. Rend. 251,* 1759.

Bláha, K., Fric, I., and Hermann, P. (1965), *Collection Czech. Chem. Commun. 30,* 304.

Brown, G. B., and du Vigneaud, V. (1941), *J. Biol. Chem. 140,* 767.

Chapeville, F., and Fromageot, P. (1961), *Biochim. Biophys. Acta 49,* 322.

Chen, P. S. (1962), Amino Acid Pools, Holden, J. T., Ed., Amsterdam, Elsevier, p 115.

Dowling, L. M., and McClaren, J. A. (1965), *Biochim. Biophys. Acta 100,* 293.

du Vigneaud, V., Brown, G. B., and Bonsnes, R. W. (1941), *J. Biol. Chem. 141,* 707.

Feigl, F. (1960), Spot Tests in Organic Analysis, Amsterdam, Elsevier, p 93.

Horn, M. J., and Jones, D. B. (1941), *J. Biol. Chem. 139,* 473.

Horn, M. J., Jones, D. B., and Ringel, S. J. (1941), *J. Biol. Chem 138,* 141.

Horn, M. J., Jones, D. B., and Ringel, S. J. (1942a), *J. Biol. Chem. 144,* 87.

Horn, M. J., Jones, D. B., and Ringel, S. J. (1942b), *J. Biol. Chem. 144*, 93.
Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.
Katzin, L. I., and Gulyas, E. (1964), *J. Am. Chem. Soc. 86*, 1655.
Kondo, Y. (1959), *J. Sericult. Sci. Japan 28*, 1.
Kondo, Y. (1962), *J. Biochem. (Japan) 51*, 188.
Massey, V., Palmer, G., and Bennett, R. (1961), *Biochim. Biophys. Acta 48*, 1.
Piez, K. A., Irreverre, F., and Wolff, H. L. (1956), *J. Biol. Chem. 223*, 687.
Rajagopal Rao, D., Ennor, A. H., and Thorpe, B. (1966), *Biochem. Biophys. Res. Commun. 22*, 163.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Rosenberg, H., Ennor, A. H., Hagermann, D. D., and Sugai, S. (1962), *Biochem. J. 84*, 536.
Rothman, F., and Higa, A. (1962), *Anal. Biochem. 3*, 173.
Schöberl, A., and Wagner, A. (1947), *Chem. Ber. 80*, 379.
Schram, E. (1963), Organic Scintillation Detectors, Amsterdam, Elsevier.
Sloane, N. H., and Untch, K. G. (1966), *Biochemistry 5*, 2658.
Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. II, London, W. Heinemann, Ltd., p 98.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stein, J. M. (1955), *Chem. Ind. (London)* 774.
Stein, J. M., and Kermack, W. O. (1959), *Biochem. J. 71*, 648.
Work, E. (1957), *Biochem. J. 67*, 416.
Wyatt, G. R. (1961), *Ann. Rev. Entomol. 6*, 75.

# Studies on the Quantum Yields of the Photodissociation of Carbon Monoxide from Hemoglobin and Myoglobin*

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ABSTRACT: The photodissociation of carbon monoxide from normal and modified myoglobin, hemoglobin subunits, and hemoglobin has been studied. The quantum yield of this process was found to be dependent, even for monomeric proteins, on ionic strength, protein concentration, and primary structure. It has not been possible to establish definitely whether or not protein aggregation state can influence quantum yield.

Carbon monoxide liganded to a heme protein is dissociated by light with a very high quantum yield (Haldane and Smith, 1896; Warburg *et al.*, 1929). This very high quantum yield for the photodissociation of carbon monoxide hemoglobin is unique to this ligand. In fact all the other ferrohemoglobin–ligand complexes, although photodissociable (Gibson and Ainsworth, 1957), have a much lower quantum yield, irrespective of their free energy of binding. As a result of measurements of the quantum yield of this photodissociation process for horse myoglobin and horse hemoglobin, Bücher and Negelein (1942) suggested that the quantum yield of the reaction might be inversely proportional to the degree of polymerization of the molecule, and hence to the number of hemes per molecule. At 4° in 0.02 M pyrophosphate buffer (pH 8.2) at a protein concentration of approximately 0.1 mg/ml, they found that the quantum yield with myoglobin was very nearly unity (0.92) while with hemoglobin it was 0.27. When the hemoglobin solution was made 2.6 M in sodium chloride, to give an ionic strength at which hemoglobin is largely dissociated into dimers, the quantum yield increased to 0.37.

An inverse relation between polymerization and quantum yield would indicate some type of intramolecular energy transfer between subunits which would be of considerable interest. In addition, if there really were a simple correlation between the quantum yield and the number of subunits per molecule, quantum yield measurements could provide a very simple and elegant means of studying the dissociation of hemoglobin at very low concentrations. With a modification of the method of Bücher and Negelein, these measurements can be extended down to protein concentrations of 0.01 mg/ml.

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